The Effect of an Intramolecular Cross-Link on Reversible Denaturation in Tryptophan Transfer Ribonucleic Acid from *Escherichia coli*†

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ABSTRACT: tRNA^{Trp} from *Escherichia coli* can be reversibly denatured and has a metastable denatured form. The effect on the interconversion has been studied of introducing a specific intramolecular restraint, namely the covalent cross-link between s^{4}U-8 and cytidine-13 produced by irradiation at 335 nm. It is found that in the cross-linked molecule both metastable conformations exist and can be interconverted under the same conditions as with the normal species; however, the equilibrium is displaced in favor of the denatured form. This is due predominantly to an increase in the rate constant for denaturation; that for renaturation is less affected. The temperature dependence of the rate constants is not significantly changed by cross-linkage. These results indicate that the denaturation does not obligatorily require the separation of bases 8 and 13, and that the presence of the cross-link does not necessitate the disruption of more structure in the formation of an effective transition state. When tRNA^{Trp} is first denatured and then irradiated, however, it cannot be cross-linked, even in the presence of Mg^{2+}. Thus, denaturation must alter slightly the conformation around the 4-thiouridine, though the thermodynamic data suggest that this disturbance is small, and hence that the two denatured species, with and without the cross-link, are largely similar but not identical in conformation.

An ordered tertiary is essential to the biological activity of tRNA. In spite of recent progress in the understanding of tRNA tertiary structure, notably through X-ray crystallography (Kim *et al.*, 1973) and nuclear magnetic resonance spectroscopy (Schulman *et al.*, 1973), there is little direct evidence about the interactions which maintain the active conformation in solution. In addition, much work suggests that a single structure will not adequately explain the behavior of tRNA in solution and indicates that some degree of polymorphism is an essential characteristic of tRNA (Thang *et al.*, 1971; Danchin and Thang, 1972; Yarus, 1972). This may be related to the great variety of interactions to which tRNA is subject in the cell but the evidence for this is at present equivocal. In investigating the interactions stabilizing tertiary structure the study of conformational transitions in tRNA assumes a special significance. The formation of a denatured conformation at low concentration of monovalent and divalent cations, favored by the decrease in electrostatic free energy, is probably a general property of tRNA (Fresco *et al.*, 1966; Goldstein *et al.*, 1972; Cole *et al.*, 1972; Webb and Fresco, 1973). The conditions of salt and temperature inducing such a transition and the thermodynamic parameters of the process of activation should, however, be expected to be highly sequence-dependent; for only comparatively few species are the denatured forms sufficiently stable to be observed by their inactivity in charging assays.

The denatured form of tRNA^{Trp} has been characterized in some detail by Ishida and Sueoka (1967, 1968a,b). The possibility that this form represents molecular aggregates has been eliminated, and hypochromicity observed on renaturation indicates that changes in base pairing or base stacking are involved. The inactive form is stable in low concentrations of salt (0.1–0.2 M NaCl), whereas high concentrations (1–2 M NaCl) favor the active form. Divalent cations are effective at much lower concentrations than monovalent ions, but are not required absolutely for the conformation of the active form. Denaturation of tRNA^{Trp} abolishes the specific interaction with Trp:tRNA ligase (Muench, 1969). Hirsch (1971) has reported that tRNA^{Trp} from *E. coli* CAJ64 (su$^+_{UGA}$) carries a single base change in the dihydrouridine stem which leads to an A·U pair instead of a G·U pair and should increase the stability of the helix in this stem. Furthermore, Hirsch found that the suppressor tRNA was not readily denatured. From these observations, and the high expected stability of the other stems which are rich in G·C pairs, it is possible that a rearrangement of the secondary structure would involve bases in the dihydrouridine stem.

In the present paper, we describe a new approach to the conformational transition in tRNA^{Trp}. To gain insight into the extent and topology of the structural rearrangement we have studied the effect on this reaction of introducing an intramolecular cross-link. Irradiation of many tRNAs from *E. coli* at 335 nm results in the formation of a covalent cross-link between 4-thiouridine-8 and cytidine-13 (Favre *et al.*, 1969). All tRNAs with this arrangement of bases appear to undergo the reaction when in their native conformation, but not under conditions which destroy tertiary structure (Favre *et al.*, 1971).

Materials and Methods

Materials. Mixed, stripped tRNA from *E. coli* B was obtained from General Biochemicals Inc. Except when used for isolation of tRNA^{Trp} it was purified by gel filtration on Sephadex G-100 (Schleich and Goldstein, 1964). It then contained 22 pmol of tryptophan acceptor capacity per A_{260} unit. Magnesium-free tRNA was obtained by heating in 50 mM

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‡ This manuscript is dedicated to Dr. E. Lederer as a tribute of my friendship for his 65th birthday.

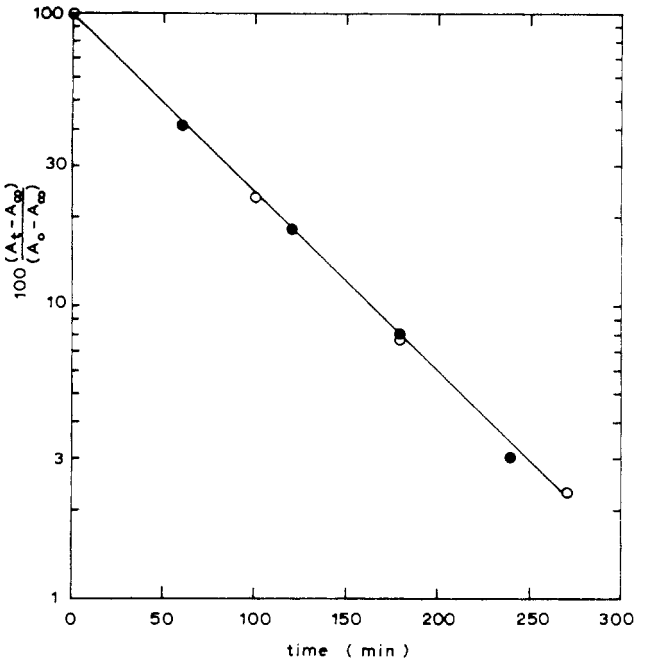

FIGURE 1: Kinetics of photoreaction between 4-thiouracil and cytosine in $tRNA^{Trp}$ (○) and unfractionated tRNA (●). The change in absorbance at 336 nm was measured as a function of time under the conditions described in the text. The abscissa expresses the difference between absorbance at time t (A_t) and after achievement of the photosteady state (A_∞) as a percentage of the total change ($A_0 - A_\infty$).

Tris-HCl (pH 8)–4 mM EDTA for 10 min at 50°. The solution was cooled and made 0.5 M in NaCl and the tRNA precipitated by addition of 2.5 volumes of cold ethanol.

$tRNA^{Trp}$ was isolated on benzoylated DEAE-cellulose essentially by the method of Joseph and Muench (1971) but omitting the final chromatography on hydroxylapatite. The tryptophan acceptor capacity of the product was 1.7 nmol/A_{260} unit.

Trp:tRNA (EC 6.1.1.2) ligase was isolated from *E. coli* B according to Joseph and Muench (1971) up to, and including, the first concentration by dialysis against polyethylene glycol. The enzyme solution was brought to 50% (v/v) in glycerol and stored at −20°. It contained 1700 units/ml.

Benzoylated DEAE-cellulose was purchased from Schwarz/Mann; Sephadex G-100 from Pharmacia; GF/C filters from Whatmann; and DL-[2-^{14}C]tryptophan (29.1 Ci/mol) from C.E.A., Saclay, France.

Charging and Discharging Assays. Charging assays were performed in 0.1 M Tris-HCl (pH 8)–5 mM $MgCl_2$–1 mM ATP–2.5 μM [^{14}C]tryptophan (29.1 Ci/mol) with excess enzyme (17 units per assay of total volume 0.1 ml). In assays conducted after incubation of tRNA in 0.5 or 0.75 M NaCl, the mixture contained 0.1 M NaCl–20 mM Tris-HCl (pH 8)–5 mM $MgCl_2$–1 mM ATP–2.5 μM [^{14}C]tryptophan (29.1 Ci/mol). Incubation was for 10 min at 25° and the reaction was terminated by cooling to 0° and adding 20 volumes of ice-cold 10% (w/v) trichloroacetic acid containing 0.2% tryptophan, and, also, in assays with purified $tRNA^{Trp}$, 10 μg/ml of tRNA as carrier. The precipitates were collected on glass fiber filters GF/C and washed successively with 10% (w/v) trichloroacetic acid containing 0.2% tryptophan, 1%, (w/v) trichloroacetic acid containing 0.2% tryptophan, and ethanol, then dried, and counted with 5 ml of scintillation fluid (0.5% 2,5-diphenyloxazole, 0.05% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene) in an Intertechnique L30 liquid scintillation spectrometer with 70% efficiency. In assays where a low background was essential, the charging reaction was stopped by the addition of 2.5 volumes of ice-cold (12:1, v/v) 95% ethanol–2 M sodium acetate buffer (pH 5) and the tryptophanyl-tRNA formed was separated from free tryptophan by centrifugation, solution in 0.1 M sodium acetate buffer (pH 5), and then reprecipitated with trichloroacetic acid, and collected as above.

Discharging assays were performed as follows: mixed tRNA or $tRNA^{Trp}$ was renatured by heating for 10 min at 50° in 0.1 M Tris-HCl (pH 8.0)–10 mM $MgCl_2$, and then charged with [^{14}C]tryptophan under the same conditions as in the charging assay above, at a concentration of about 2 nmol of $tRNA^{Trp}$/ml. The product was isolated by phenol extraction and ethanol precipitation (Maxwell *et al.*, 1968) and redissolved in 0.1 M sodium acetate buffer (pH 4.1) or 0.1 M sodium acetate buffer (pH 5.6) containing 4 mM EDTA, and incubated for 20 min at 37° to produce active or inactive forms (Gartland and Sueoka, 1966). After precipitation with 2.5 volumes of ethanol at −20°, the tRNA was dissolved in discharging buffer: 0.1 M Tris-HCl (pH 7.2)–5 mM $MgCl_2$–4 mM AMP–4 mM pyrophosphate, and aliquots (0.1 ml, containing about 13 pmol of tryptophanyl-$tRNA^{Trp}$) were incubated for 1–10 min at 37° with 17 units of enzyme. The tRNA was precipitated, collected, and counted as in the charging assay above.

tRNA (s^4U-*C*).[1] tRNA (s^4U-C) was produced by irradiation at 335 nm in 0.1 M Tris-HCl (pH 8.0)–10 mM $MgCl_2$ for 5 hr at 20° with a Bausch and Lomb monochromator equipped with an HBO 200-W super-pressure mercury lamp, as described by Favre *et al.* (1971). The tRNA was renatured before irradiation by heating for 10 min at 50°. To determine the relative rates of photoreaction of active and inactive $tRNA^{Trp}$, irradiation was performed with the Cunow lantern at 5–10° as described by Berthelot *et al.* (1972), except that the above buffer was used and that after irradiation but before reduction with $NaBH_4$ the samples were renatured by heating for 10 min at 50°. Aliquots containing 0.15 A_{260} unit were taken for reduction and determination by fluorescence measurements of the reduced thiouridine–cytidine photoproduct, with a Jobin et Yvon spectrofluorimeter. In other experiments the thiouridine–cytidine cross-link was determined by spectrophotometry after reduction with 10 mM $NaBH_4$ (pH 9.7), as described by Favre and Yaniv (1971).

Results

Formation of the Cross-Link in Native $tRNA^{Trp}$. Irradiation of $tRNA^{Trp}$, renatured by heating in the presence of Mg^{2+}, produced changes in the absorption spectrum of 4-thiouridine between 305 and 380 nm, which were quantitatively more marked than in $tRNA_1^{Val}$ (Favre *et al.*, 1971). A reduction of 41% in the absorption at 335 nm and the appearance of a shoulder at about 355 nm led to a spectrum very similar to that of cross-linked $tRNA_1^{Val}$ but with greater hypochromicity. The kinetics of the modification were first order, and similar in rate constant to those of unfractionated tRNA from *E. coli* (Figure 1). Reduction with sodium borohydride of the photoproduct in $tRNA^{Trp}$ led to a new product with spectral and fluorescence properties similar to those seen in $tRNA_1^{Val}$ (Favre and Yaniv, 1971), providing good evidence for the formation of the same cross-link upon irradiation.

[1] Abbreviation used is: $tRNA^{Trp}$ (s^4U-C), $tRNA^{Trp}$ bearing the 4-thiouridine–cytidine cross-link.

TABLE I: Stability of 4-Thiouridine–Cytidine Photoproduct under Experimental Conditions.[a]

Conditions of Treatment			
Buffer	Temp (°C)	Time (min)	(A_{384}/A_{260}) × 100
No treatment			1.47
0.1 M Sodium acetate buffer (pH 4.1)	37	20	1.41
0.1 M Sodium acetate buffer (pH 5.6)	37	20	1.43
0.1 M Tris-HCl (pH 8)–5 mM $MgCl_2$	37	20	1.45
0.1 M Tris-HCl (pH 8)–5 mM $MgCl_2$	50	10	1.45
0.1 M Tris-HCl (pH 8)–5 mM $MgCl_2$ (nonirradiated)	50	10	0.30

[a] tRNA (s^{4}U-C) was incubated under the stated conditions and then reduced with $NaBH_4$ at pH 9.7 as described in the text. After precipitation with ethanol the tRNA was redissolved in 0.1 M Tris-HCl (pH 8.0)–5 mM $MgCl_2$ for determination of the absorbance at 260 and 384 nm.

In the standard assay, tRNA^{Trp} (s^{4}U-C) could be charged with tryptophan to the same level, 1700 pmol/A_{260} unit, as the normal species.

Conversion of Tryptophanyl-tRNA^{Trp} (s^{4}U-C) and tRNA^{Trp} (s^{4}U-C) to an Inactive Form. The inactive form of normal tRNA^{Trp}, charged or uncharged, can be produced by incubation at 37° in sodium acetate buffer (pH 5.6) in the absence of magnesium ions (Gartland and Sueoka, 1966). We investigated whether a similar transition could be brought about when the molecule was partially constrained by the 4-thiouridine–cytidine cross-link. Acylated tRNA^{Trp}, for which the forms are more stable in sodium acetate buffer than those of the uncharged molecule (Muench, 1969), was incubated under the above conditions and tested as a substrate for tryptophanyl-tRNA ligase in the presence of AMP and PP_i to promote enzymatic discharge. The treatment at pH 5.6 produced a form resistant to discharge, no matter whether or not the cross-link was present. As shown in Figure 2, after a rapid initial discharge representing about 20% of the tryptophanyl-tRNA, the inactive preparations were discharged at a rate characteristic of nonenzymic hydrolysis. The active preparations, made by incubation at pH 4.1, were substantially discharged within 1 min.

The integrity of the cross-link throughout the experiment was shown by subsequent reactivity with sodium borohydride, giving the absorption spectrum of the reduced photoproduct. This absorbs characteristically at 384 nm, and the ratio of this absorbance to that at 260 nm, as shown in Table I, was not significantly changed in material subjected to the treatments described.

Similar experiments were performed with uncharged tRNA^{Trp} (s^{4}U-C), but using subsequent acceptor capacity as the assay for the proportions of active and inactive forms. Again it was found (Table II) that an inactive form could be made with the cross-linked species since treatment at pH 5.6 reduced the tryptophan acceptor capacity of either mixed tRNA or purified tRNA^{Trp} to a low level.

Renaturation of Inactive tRNA^{Trp} (s^{4}U-C) in the Presence of Mg^{2+}. The active form of tRNA^{Trp} can be regenerated from the inactive form by heating to 50° in 0.1 M Tris-HCl (pH 8.0)–5 mM $MgCl_2$. Under these conditions, equilibrium lies almost completely on the side of the active form and the transition exhibits first-order kinetics (Ishida and Sueoka, 1968a).

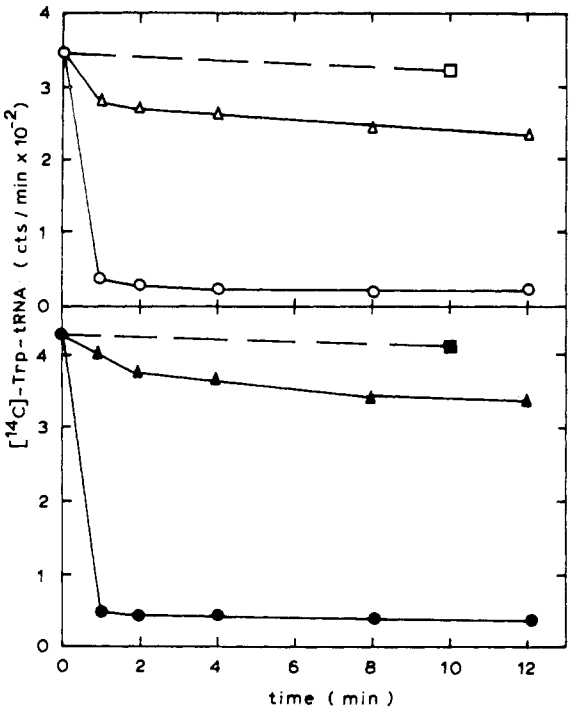

FIGURE 2: Enzymatic discharge of active and inactive Trp-tRNA (open symbols) and Trp-tRNA (s^{4}U-C) (filled symbols). Active and inactive forms were made by incubation at pH 4.1 or 5.6, and aliquots containing 8 pmol of esterified [^{14}C]]tryptophan (29 mCi/mol) were incubated for the time indicated in 0.1 M Tris-HCl (pH 7.2)–5 mM $MgCl_2$–4 mM AMP–4 mM pyrophosphate, with or without Trp:tRNA ligase, as described in the text. The figures show the [^{14}C]tryptophan remaining esterified to the active form (○) and the inactive form (△) in the presence of enzyme, and to either form with no enzyme (□).

The cross-linked and non-cross-linked inactive forms of tRNA^{Trp} were compared with respect to the extent of renaturation achieved by incubation for 10 min at temperatures between 25 and 55°. The renaturation curves showed no significant effect of the cross-link on renaturation (Figure 3). The cross-link was found to be stable to heating at 50° by the criteria previously described (Table I). From the rate constants and their temperature dependence a value of 78 kcal mol^{-1} is obtained for ΔH_r° *, the enthalpy change for activation and renaturation, and 23 kcal mol^{-1} and 175 cal deg^{-1} mol^{-1} for ΔG_r° * and ΔS_r° *, calculated as described in Table III.

TABLE II: Tryptophan Acceptor Capacity of tRNA and tRNA (s^{4}U-C) after Treatment at pH 4.1 or 5.6.

Material	Acceptor Capacity (pmol/A_{260} unit) after Treatment at pH 4.1	pH 5.6
tRNA	18.8	2.7
tRNA (s^{4}U-C)	19.5	2.8
tRNA^{Trp}	1730	193
tRNA^{Trp} (s^{4}U-C)	1680	180

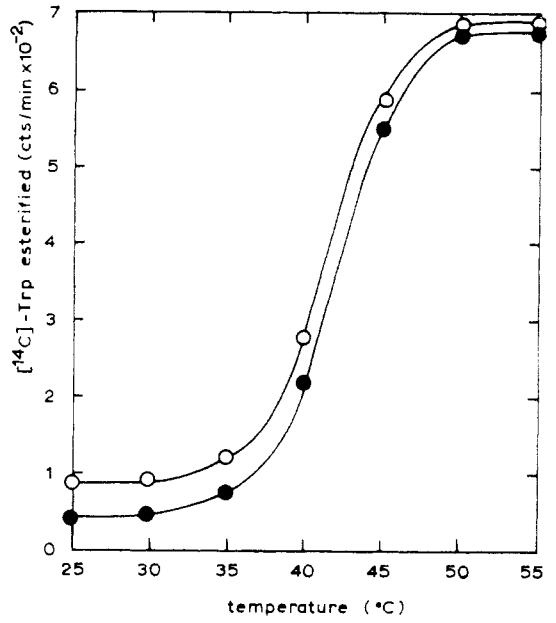

FIGURE 3: The effect of temperature on the renaturation of $tRNA^{Trp}$ (○) and $tRNA^{Trp}$ (s^4U-C) (●). The inactive forms were made by incubation at pH 5.6 of tRNA and tRNA (s^4U-C). Aliquots of each containing about 16 pmol of potential tryptophan acceptance were incubated for 10 min in 0.1 M Tris-HCl (pH 8–5 mM $MgCl_2$, and assayed at 25° for tryptophan acceptance. Further details are in the text.

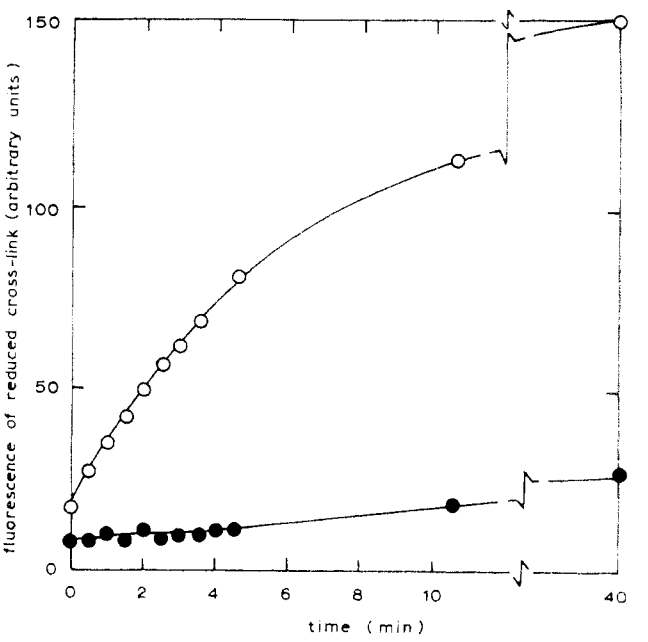

FIGURE 4: Kinetics of photoreaction between 4-thiouracil and cytosine in active (○) and inactive (△) $tRNA^{Trp}$. Active and inactive forms were made by incubation at pH 4.1 or 5.6 and were irradiated simultaneously; 0.15 A_{260} unit was taken at intervals, renatured by heating, and reduced with $NaBH_4$. The reduced photoproduct was measured by fluorescence at 450 nm, excited at 390 nm.

Irradiation of Denatured $tRNA^{Trp}$ at 335 nm. Although no difference had yet been shown between $tRNA^{Trp}$ and $tRNA^{Trp}$ (s^4U-C) during denaturation or renaturation, there was a possibility that the two inactive forms, with and without the cross-link, had different conformations around the 4-thiouridine. We examined, therefore, whether inactive $tRNA^{Trp}$ would undergo the photoreaction when irradiated at 335 nm. The experiment was conducted under conditions favoring the active conformation, namely 5 mM Mg^{2+}, in which the inactive form is metastable. The course of the reaction was followed by measuring the fluorescence of the reduced photoproduct (Figure 4). Inactive $tRNA^{Trp}$ was used as a control, and in this case a logarithmic reaction plot (not shown, but analogous to Figure 1) revealed a first-order reaction with rate constant 2.1×10^{-3} sec^{-1}. By contrast, almost no photoreaction was observed with the inactive form of $tRNA^{Trp}$ (Figure 4). There is, therefore, a difference in the orientation of 4-thiouridine-8 and cytidine-13 in the denatured conformation, though the change might be quite small as the photoreaction appears to be a very sensitive probe of the relative orientation of these bases. In these experiments the tRNA was renatured by heating after irradiation but before reduction so the observed difference cannot be due to differing rates of reaction between the photoproduct and sodium borohydride or a lower quantum yield of fluorescence in active reduced $tRNA^{Trp}$ (s^4U-C).

Equilibrium between Native and Denatured Forms in the Absence of Mg^{2+}. The experiments so far described had made clear that the cross-link did not prevent access to an inactive form, but did affect, though to an unknown extent, the conformation of this form. There was, however, no significant change in a set of thermodynamic parameters associated with renaturation. In order to gain some knowledge of the extent to which the cross-link might modify the processes of interconversion, we investigated these more fully in conditions under which the equilibrium constant and the kinetics of approach to equilibrium could be readily measured. tRNA and tRNA (s^4U-C) were obtained free from magnesium ions, and the fraction of active form after the attainment of equilibrium was measured throughout a range of salt concentrations.

TABLE III: Thermodynamic Equilibrium and Activation Parameters for the Interconversion of Native and Denatured Forms.[a]

	0.5 M NaCl		0.75 M NaCl	
tRNA	Normal	Cross-linked	Normal	Cross-linked
ΔG_d°	−0.37	−0.88	0.63	−0.02
ΔH_d°	35	31	34	31
ΔS_d°	114	103	107	97
ΔG_d° *	22.78	22.10	23.70	23.09
ΔH_d° *	94	95	93	96
ΔS_d° *	230	234	224	235
ΔG_r° *	23.15	22.98	23.07	23.11
ΔH_r° *	59	64	59	65
ΔS_r° *	116	131	117	138

[a] Thermodynamic parameters are shown for denaturation and renaturation of normal and cross-linked $tRNA^{Trp}$ in 0.5 and 0.75 M NaCl–50 mM Tris-HCl (pH 8.0)–1 mM EDTA, and are obtained from the data of Figures 6–8. Free energy changes (at 37°) and enthalpy changes are in kcal mol^{-1} and entropy changes are in cal deg^{-1} mol^{-1}, and were calculated from the relations: $\Delta G_d^\circ = -RT \ln K$, $\Delta G_d^\circ * = -RT \ln (k_1 h/kT)$, $\Delta G_r^\circ * = -RT \ln (k_{-1} h/kT)$, $\Delta H_d^\circ = -R \, d \ln K/d(1/T)$, $\Delta H_d^\circ * = -R \, d \ln k_1/d(1/T) - RT$, $\Delta H_r^\circ * = -R \, d \ln k_{-1}/d(1/T) - RT$, $\Delta S_d^\circ = (\Delta H_d^\circ - \Delta G_d^\circ)/T$, $S_d^\circ * = (\Delta H_d^\circ * - \Delta G_d^\circ *)/T$, $\Delta S_r^\circ * = (\Delta H_r^\circ * - \Delta G_r^\circ *)/T$, where h is Planck's constant and k is the Boltzman constant.

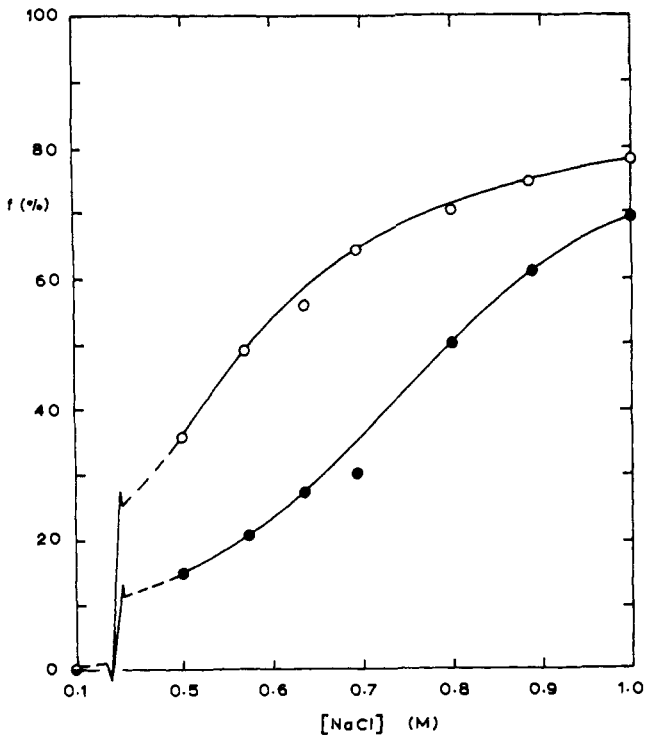

FIGURE 5: Proportion of active form in tRNA^{Trp} (○) and tRNA^{Trp} (s^{4}U-C) (●) at equilibrium. Mixed tRNA and tRNA (s^{4}U-C) were freed from Mg^{2+} as described in the text, and aliquots containing 0.4 A_{260} unit were incubated in 50 mM Tris-HCl (pH 8.0)–1 mM EDTA and NaCl at the molarity indicated (total volume 20–30 μl) for 3 hr at 38°. Tryptophan acceptance was assayed as described in the text, and is expressed as a percentage (f) of that of aliquots renatured at 50° for 10 min in 0.1 M Tris-HCl (pH 8.0)–10 mM $MgCl_2$.

From the results (shown in Figure 5) it can be seen that at all points between 0.5 and 1.0 M NaCl a smaller proportion of active form was present in the case of the cross-linked sample. The effect of cross-linkage therefore was to displace the equilibrium in favor of the denatured form.

The variation of K, the equilibrium constant, with temperature was determined at two concentrations of NaCl: 0.5 and 0.75 M. It was found that, within experimental error, there was a parallel displacement of the linear relationship between log K and temperature as a result of cross-linkage at either salt concentration (Figure 6). Neither of these changes, therefore, significantly alters the enthalpy of denaturation, but each must affect the rate constants for denaturation or renaturation or both.

Kinetics of Interconversion. In order to obtain thermodynamic data on the processes of activation involved in denaturation and renaturation, we measured the rates of approach to equilibrium at different temperatures and two concentrations of NaCl. Predominantly active or inactive tRNA and tRNA (s^{4}U-C) was obtained by incubation at 50° in 0.1 M NaCl or 2 M NaCl, respectively and cooled to 0°, and the concentration of NaCl then adjusted to 0.5 or 0.75 M. The rates of change of tryptophan acceptor capacity are shown in Figure 7 and were found to be first order. In many cases it was possible to follow both denaturation and renaturation under the same conditions of salt concentration and temperature, and it was found then that the data were consistent with a single rate constant.

Hence we have treated the interconversion as a reversible first order reaction and derived the rate constants k_1 and k_{-1}

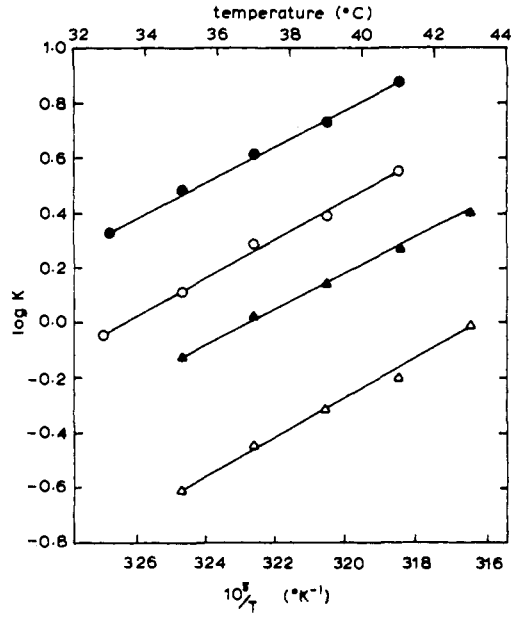

FIGURE 6: Equilibrium constants for reversible denaturation as a function of temperature. Equilibrium constants were determined by tryptophan acceptance assays as described in the text, for tRNA^{Trp} (○) and tRNA^{Trp} (s^{4}U-C) (●) in 0.5 M NaCl, and tRNA^{Trp} (△) and tRNA^{Trp} (s^{4}U-C) (▲) in 0.75 M NaCl. Solutions contained also 50 mM Tris-HCl (pH 8.0)–1 mM EDTA. K is measured as the ratio of the fraction of inactive form over the fraction of active form, at equilibrium.

for denaturation and renaturation, respectively, which are presented as functions of temperature on Figure 8. They are seen as families of nearly parallel lines and show that the major effect of either cross-linkage or decrease in salt concentration is to increase k_1 by a factor independent of temperature. The process of renaturation is much less affected. The thermodynamic parameters describing the processes of ac-

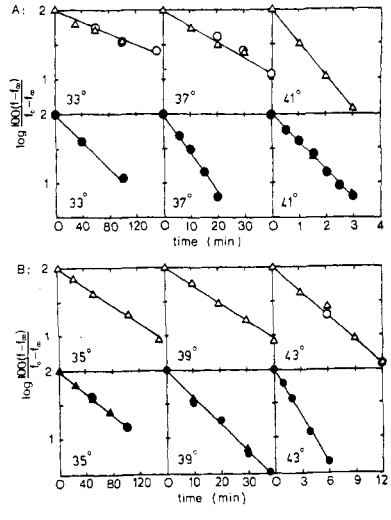

FIGURE 7: Kinetics of approach to equilibrium between native and denatured forms. Active and inactive forms were obtained by incubation at 50° in 2.0 and 0.1 M NaCl, respectively, and the fraction of tRNA in the active form, f, was measured by acceptance assay. f_0 and f_∞ are the values of f at time zero and after attainment of equilibrium. Approach to equilibrium was followed for active (○) and inactive (△) tRNA^{Trp} and active (●) and inactive (▲) tRNA^{Trp} (s^{4}U-C), in both 0.5 M (A) and 0.75 M (B) NaCl–50 mM Tris-HCl (pH 8.0)–1 mM EDTA, at the temperatures indicated.

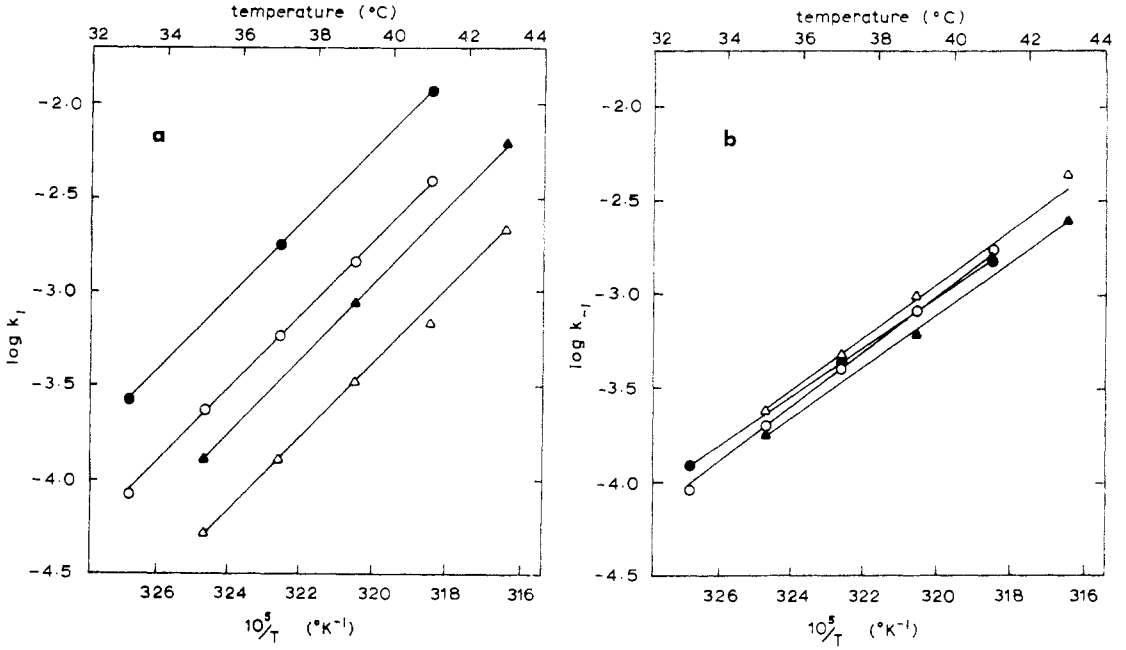

FIGURE 8: Rate constants for denaturation (k_1) and renaturation (k_{-1}). k_1 (a) and k_{-1} (b) are calculated from the data of Figure 6 and 7, using the relations $\log (f - f_\infty)/(f_0 - f_\infty) = 0.4343(k_1 + k_{-1})t$ and $K = k_1/k_{-1}$ for $tRNA^{Trp}$ (○) and $tRNA^{Trp}$ (s^4U-C) (●) in 0.5 M NaCl, and $tRNA^{Trp}$ (△) and $tRNA^{Trp}$ (s^4U-C) (▲) in 0.75 M NaCl. k_1 and k_{-1} are in sec^{-1}.

tivation of denaturation and renaturation, obtained from the temperature dependence and magnitude of k_1 and k_{-1} by transition-state theory (Eyring, 1935), are little affected by the presence of the cross-link (Table III).

In considering quantitative differences between the species, it is necessary to discuss the effect of partial cross-linkage in the samples of $tRNA^{Trp}$ and incomplete cross-linkage in $tRNA^{Trp}$ (s^4U-C). The measurements were made with mixed *E. coli* tRNA, and characterization by fluorescence after reduction with $NaBH_4$ indicated that less than 3% of the 4-thiouridine in the batches used was cross-linked. Estimation of the $tRNA^{Trp}$ remaining non-cross-linked after irradiation is more difficult. Three factors could be involved: incomplete modification of uridine-8 *in vivo*, a residual level of inactive form remaining during irradiation, and the possible attainment of a photosteady state during irradiation. The combined effect of these is difficult to measure, but is unlikely to lead to more than 15% contamination of tRNA (s^4U-C) by $tRNA^{Trp}$ and probably much less.

Ishida and Sueoka (1968a) have previously described the kinetics of interconversion in $tRNA^{Trp}$ and reported thermodynamic parameters. One set of conditions, in 0.5 M NaCl, is common to their and our experiments and the respective data should be compared.

Reasonable agreement exists between our observations and rate constants calculated from the renaturation data of Ishida and Sueoka. Their denaturation data, however, though showing approximately similar temperature dependence and hence enthalpy parameters, yield rate constants nearly one order of magnitude higher than ours. It seems to us that data of Ishida and Sueoka are not consistent with a reversible first-order reaction, whereas ours are. The major difference between their experiments and those described here is that our solutions contained a low concentration of EDTA to control contamination by divalent metal ions. It is possible that this is related to the discrepancies between rate constants. Nevertheless, there exists reasonable agreement between the thermodynamic parameters reported by Ishida and Sueoka (1968a) and those we report here.

Discussion

We have described the effects of a specific intramolecular restraint on a reversible conformational change in $tRNA^{Trp}$. Some insight into the mechanism of denaturation can be drawn from these observations. It has been shown that both native and denatured forms of $tRNA^{Trp}$ (s^4U-C) exist and can be interconverted under conditions effective with the non-cross-linked form. The question must be considered of the extent to which the course of denaturation has been modified by the introduction of the cross-link. The native conformation of $tRNA_1^{Val}$ is very little perturbed when cross-linked (Favre *et al.*, 1971). The photoreaction has been investigated in many tRNA species possessing 4-thiouridine-8 and cytidine-13, and the surprising constancy in the kinetic parameters suggests a conformation in that region which is very strongly conserved (A. Favre and G. Thomas, unpublished work). This applies also to the active conformation of $tRNA^{Trp}$. The absence of photoreaction in the inactive conformation, even in the presence of Mg^{2+}, implies that some change in the relative orientation of the two bases involved must accompany denaturation. However, the thermodynamic parameters associated with denaturation and renaturation show no significant effect of the cross-link. We find similar values for ΔH_d° * in $tRNA^{Trp}$ and $tRNA^{Trp}$ (s^4U-C) and conclude that the formation of an effective transition state requires breakage of about the same number of stacking interactions in each case, and probably rupture of the same base pairs. The activation energies observed for denaturation and renaturation appear too high to be accounted for by loss of tertiary structure and imply that considerable structural disorganization must take place in the transition. More specific evidence about denaturation comes from the mutant $tRNA^{Trp}$ (CAJ64 su^+). In this species the base change G (24) → A

enhances the stability of the native form (Hirsch, 1971) and suggests that the dihydrouridine stem is involved in the transition state, if not actually in a rearranged secondary structure of the denatured form. By applying the criteria developed by Gralla and Crothers (1973) one may deduce that in the absence of further stabilizing features (tertiary structure) the dihydrouridine stem of wild-type $tRNA^{Trp}$ is not stable, in contrast with that of $tRNA^{Trp}$ (CAJ64 su^+). Therefore the maintainance of base pairing in this stem in wild-type $tRNA^{Trp}$ is probably coupled to an element of tertiary structure, perhaps involving the colinear stacking of this stem with the anticodon stem.

Ishida and Sueoka (1968a) concluded that a pair of phosphate groups close to each other was required for conversion of $tRNA^{Trp}$ to the active form; the role of cations in renaturation was to permit the close approach of these groups. This receives some support from the very high dependence of the mid-transition temperature for denaturation upon cation concentration. It should be recalled that the dependence of the equilibrium constant on ionic strength arises through changes in k_1, the rate constant for denaturation; the reverse process is substantially independent of salt concentration.

Two extreme interpretations can be presented for our observations. One possibility is that the region of tertiary structure around bases 8 and 13 in the native form is substantially, though not absolutely, conserved in the denatured conformation. One mechanism at least for such a hypothesis can be envisaged, which involves the re-pairing of some bases from the disrupted dihydrouridine stem with bases in the extra loop, so as to make an alternative stem which stacks on top of the anticodon stem, if a single base bulge defect is permitted.

The alternative interpretation is that bases 8 and 13 are in a totally unstructured region in the denatured form. A few such structures, analogous to those suggested by Cole *et al.* (1972), can be drawn for $tRNA^{Trp}$, which leave bases 8 and 13 in an unstructured loop region. Excluded by our experiments is a range of possibilities for the denatured form in which the introduction of the (s^4U-C) cross-link would entail disruption of structure. These hypotheses and the models for the denatured form which they suggest will be considered in more detail elsewhere with the presentation of data on the denaturation of $tRNA^{Trp}$ (su^+_{UGA}CAJ64).

Two final points deserve comment. Studies of denaturation in other species have revealed certain common features. Notably, changes in accessibility to complementary oligonucleotides or specific nucleases implicate the dihydrouridine stem and loop and the "extra arm" as regions modified in reversible denaturation (Streeck and Zachau, 1971, 1972; Uhlenbeck *et al.*, 1972). Such changes would be in keeping with the hypothesis proposed above. More intriguing are the effects of denaturation on the accessibility of the anticodon loop, which are revealed by both types of study. These may be explained by the direct masking of the region by interaction with another region of the molecule (Streeck and Zachau, 1972). A further possibility is that the conformation of the anticodon is sensitive to structural change in another part of the molecule. In the latter case, an interesting parallel may be presented by the mechanism of suppression by $tRNA^{Trp}$ (CAJ64, su^+).

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